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Award Number: W81XWH-08-1-0044

TITLE: Epinephrine-Induced and Antiapoptotic Signaling in Prostate Cancer

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REPORT DATE: May 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 31-05-2009		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 MAY 2008-30 APR 2009	
4. TITLE AND SUBTITLE Epinephrine-Induced and Antiapoptotic Signaling in Prostate Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-08-1-0044	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) George Kulik Email: gkulik@wfubmc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Wake Forest University Health Sciences Winston-Salem, NC, 27157				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT We hypothesize that epinephrine released after emotional stress activates anti-apoptotic signaling pathways in prostate tumors, and contributes to the resistance of advanced prostate cancer to therapies. To test this hypothesis, we propose to examine if stress/epinephrine induces the activation of PKA and BAD phosphorylation in prostate tumors; and determine if stress/epinephrine reduces therapeutic efficacy of anti-cancer treatments. We have developed methodologies to test the role of β 2-AR in stress-induced activation of PKA. PKA activity could be judged by phosphorylation of CREB and BAD. Although BAD phosphorylation is more relevant to anti-apoptotic effect of epinephrine, CREB could be used as biomarker of PKA activation. Still, additional experiments are needed to reliably monitor activation of PKA and BAD phosphorylation in xenograft tumors <i>in vivo</i> . Since C42 cells are PTEN deficient, PI3K/Akt pathway is constitutively active and BAD is constitutively phosphorylated in these cells. Thus, to determine whether BAD could be phosphorylated by stress/epinephrine <i>in vivo</i> we need to inhibit PI3K in C42 xenografts. To accomplish this two approaches are under way: first, to use new PI3K inhibitor ZSTK474, second to generate cells that inducibly express PTEN phosphatase.					
15. SUBJECT TERMS Prostate cancer, apoptosis, epinephrine, PKA; BAD					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT U U	18. NUMBER OF PAGES 30	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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Introduction

Prostate cancer patients reportedly show increased levels of cancer fear and mood disturbances compared to other cancer patients. However, information regarding the effects of stress hormones on prostate cancer cells, and on responses of prostate tumors to therapies, is limited.

We hypothesize that epinephrine released after emotional stress activates anti-apoptotic signaling pathways in prostate tumors, and contributes to the resistance of advanced prostate cancer to therapies. To test this hypothesis, we propose to examine if stress/epinephrine induces the activation of PKA and BAD phosphorylation in prostate tumors; and determine if stress/epinephrine reduces therapeutic efficacy of anti-cancer treatments.

During the first year (June 2008-May 2009) we proposed to conduct following experiments:

Months 1-12

Test if stress/epinephrine activates PKA and increases BAD phosphorylation in subcutaneous xenografts.

Months 6-18

Test if stress/epinephrine activates PKA and increases BAD phosphorylation in xenografts implanted in bones.

Body

1. Stress/Epinephrine-induced PKA activation and Bad phosphorylation in subcutaneously implanted prostate cancer xenografts.

In recent publication we have shown that epinephrine protects prostate cancer cells from apoptosis by activating PKA that in turn phosphorylates BAD (1).

The task 1 of SOW was to determine whether subjecting mice to emotional stress will induce PKA activation and BAD phosphorylation in prostate cancer xenografts and also to test whether effects of stress are mediated via beta 2 adrenoreceptors (β 2-AR).

To determine whether effects of stress on PKA activation BAD phosphorylation and apoptosis are mediated via epinephrine/ β 2-AR we planned to use antagonists of β 2-AR: propranolol and ICI118,551.

Experiments were planned as follows:

- 1) test whether β 2-AR antagonists (propranolol or ICI118,551) will block anti-apoptotic effect of epinephrine and BAD phosphorylation induced by epinephrine in C42Luc cells (this is positive control for in vivo experiments with C42Luc xenografts)
- 2) examine status of CREB phosphorylation in C42Luc xenografts
- 3) examine status of BAD phosphorylation in C42Luc xenografts
- 4) test whether inhibitors of PI3K will lead to AKT and BAD dephosphorylation in C42Luc xenografts
- 5) test whether stress or epinephrine injection will restore BAD phosphorylation in C42Luc xenografts treated with PI3K inhibitors.

1. 1) Test whether β 2-AR antagonists (propranolol or ICI118,551) will block anti-apoptotic effect of epinephrine and BAD phosphorylation induced by epinephrine in C42Luc cells (positive control for in vivo experiments with C42Luc xenografts).

Experiments with β 2-AR selective antagonist ICI118,551 have shown that it inhibits anti-apoptotic effects of epinephrine but not of vasoactive intestinal peptide that acts via VPAC1 receptors nor the anti-apoptotic effect of forskolin, a direct activator of adenylyl cyclase (Fig.1 A). Consistently, pre-treatment with propranolol prevented epinephrine-induced phosphorylation of both CREB and BAD (Fig.1B lanes 4,6,9). These experiments also showed that in C42 cells that grow in either serum free media or in the presence of serum, BAD is constitutively phosphorylated and only minor increase over background phosphorylation could be detected after epinephrine treatment (compare lane 1 with lanes 3 and 5). Pre-

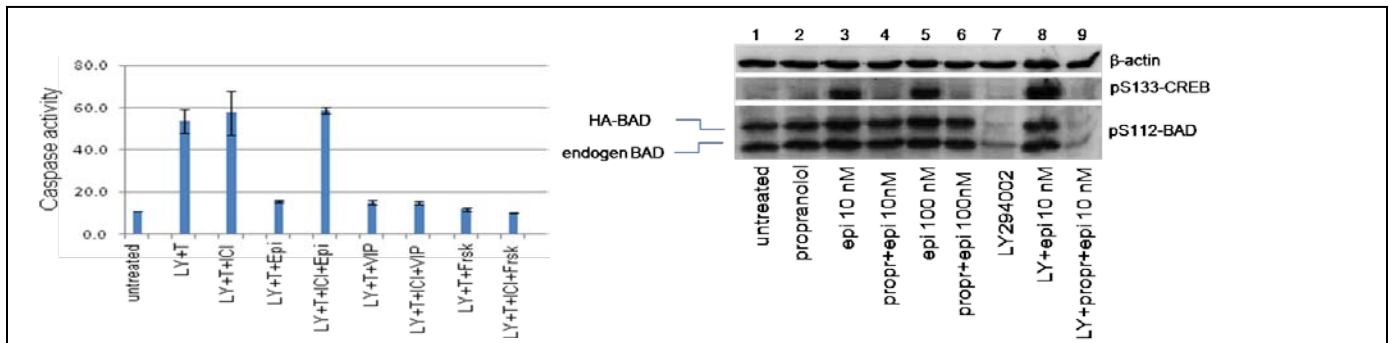


Fig. 1. Antagonists of β 2-AR inhibit epinephrine-induced survival and phosphorylation of CREB and BAD.

a) Cells were put in serum free media and treated with apoptosis-inducing agents (combination of LY294002 and thapsigargin) alone or in combination with ICI118,551 (ICI, 10 μ M) where indicated, followed by epinephrine (Epi, 100nM), Vasoactive intestinal peptide (VIP, 100nM), Forskolin (Fsk, 5 μ M). Three hours later cells were collected and caspase activity measured with fluorescent substrate DEVD-afc.

b) Cells were pretreated with 10 μ M of propranolol alone or in combination with LY294002, followed by epinephrine. One hour later cells were lysed and phosphorylation of CREB and BAD was examined by Western blotting.

treatment with PI3K inhibitor LY294002 completely abolished BAD phosphorylation at S112; while epinephrine added 1h after LY294002 restored BAD phosphorylation. Epinephrine-induced BAD phosphorylation was blocked in the presence of 10 μ M of propranolol (Fig.1B lanes 7,8,9).

These results led us to following conclusions:

- Propranolol could be used to inhibit activation of β 2-AR/PKA/BAD pathway in C42 cells by epinephrine. If propranolol will inhibit stress-induced activation of PKA and BAD phosphorylation in C42 xenografts in vivo it will confirm that effect of stress are mediated via β 2-AR.
- Effects of stress/epinephrine on BAD phosphorylation in C42 xenografts should be studied in the presence of PI3K inhibitors that eliminate constitutive BAD phosphorylation.
- Phosphorylation of ectopically expressed HA-BAD mirrors endogenous BAD phosphorylation, thus analysis of HA-BAD allows adequately interpret modifications of endogenous BAD.
- Other GPCR agonists may potentially activate PKA/BAD pathway and protect prostate cancer cells from apoptosis.

1. 2) Examine status of CREB phosphorylation in C42Luc xenografts

Our pilot data have shown that in C42Luc xenografts CREB is not phosphorylated unless mice are subjected to emotional stress (Fig.2 A).

However, more extensive analysis of CREB phosphorylation in C42Luc xenografts showed that in some cases CREB was phosphorylated in tumors of mice that were not subjected to stress (not shown). Furthermore we observed substantial variations in CREB phosphorylation in mice subjected to stress with or without prior injection of propranolol (Fig. 2B). This is a striking discord with our tissue culture data, that consistently show CREB phosphorylation when cell are treated with agonists that activate PKA, and lack of epinephrine-induced CREB phosphorylation in cells pretreated with propranolol (Fig. 1B).

Subsequent measurements of epinephrine in blood collected from mice showed that response to stress is significantly varied between individual mice. Even gentle handling triggers epinephrine spike in some mice while others may not respond to stressful treatments. Since propranolol will prevent activation of β 2-AR/PKA pathway only if injected prior to stress, CREB could be phosphorylated even in mice injected with propranolol. To reduce stress from handling we plan to handle mice daily for at least a week prior to follow-up experiments. We also perfected in-house epinephrine measurements in mouse blood by ELISA. Pilot experiments have shown that continuous handling throughout the week combined with isoflurane anesthesia prior to intratumoral injections permits keeping epinephrine levels below 1nM in

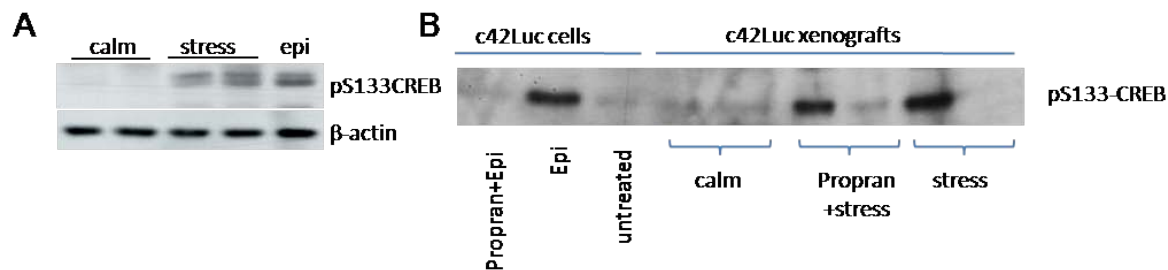


Fig.2 CREB phosphorylation in C42 xenografts.

a) Mice with subcutaneous C42 xenografts were left intact, subjected to stress by immobilization and exposure to fox scent or injected with epinephrine. 40 min later tumors were excised and phosphorylation of CREB was examined by Western blotting.

b) Mice with subcutaneous C42 xenografts were injected with 100 μ l of 10 mM propranolol (where indicated) followed by stress. 40 min later tumors were excised and phosphorylation of CREB was examined by Western blotting.

60% mice. These data indicate that assignment of tumor samples for analysis of signaling pathways should be done based on blood epinephrine measurements.

Given the individual variability in stress response, the lack of tools that allow continuous monitoring activation of PKA pathway in xenograft tumors poses a formidable problem. To create such tool we decided to generate C42 cells that express luciferase under control of CRE response element. In order to increase CRE-driven expression of luciferase to the level that permit detecting luminescence in xenografts *in vivo* we used GAL-VP16 two-step transcriptional amplification system (2). Generation of cell lines that stably express CRE-driven luciferase is currently under way. We anticipate that noninvasive monitoring of CRE reporter combined with biochemical analysis of CREB phosphorylation will permit a comprehensive analysis of stress-induced activation of PKA pathway in C42 xenografts *in vivo*.

1.3) Examine status of BAD phosphorylation in C42Luc xenografts.

Analysis of BAD phosphorylation in C42Luc xenografts showed that BAD is constitutively phosphorylated in xenografts *in vivo* (Fig. 3A)

1. 4) Test whether inhibitors of PI3K will lead to AKT and BAD dephosphorylation in C42Luc xenografts.

In contrast to results obtained with C42 cells in tissue culture that show parallel reduction of phosphorylation of both Akt and BAD (3, 4), after injections of LY294002 into xenograft tumors BAD remained phosphorylated despite Akt phosphorylation was substantially reduced (Fig. 3A)

Analysis of the dynamics of Akt phosphorylation in xenografts tumors has shown that Akt remains dephosphorylated for only 40 min, unlike in tissue culture, where Akt remains dephosphorylated for several hours. Based on tissue culture experiments BAD is expected to be de-phosphorylated within 2 hours after Akt de-phosphorylation (Sastry et al, JBC 281, pp. 20891–20901supplem. figure 1) To maintain PI3K/Akt pathway continuously inhibited for several hours we repeatedly injected LY294002 into C42LucBAD xenografts. Still, BAD remained phosphorylated even 3h after LY294002 injections began. This result suggests that BAD de-phosphorylation *in vivo* could take longer than in tissue culture. To maintain PI3K/Akt pathway inhibited for longer period of time we plan to substitute LY294002 with another PI3K inhibitor ZSTK474. Pilot experiments have shown that ZSTK474 inhibits PI3K and induces apoptosis at 0.5 μ M which is 40 fold lower concentration than LY29002 (Fig. 3B). *In vivo* testing of ZSTK474 is currently ongoing.

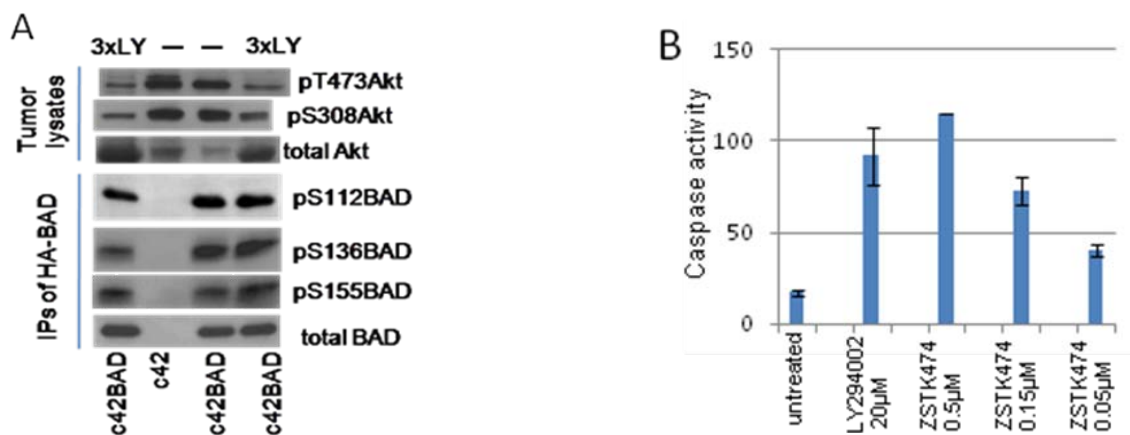


Fig. 3. Effects of PI3K inhibitors on phosphorylation of Akt, BAD and apoptosis.

- A) Xenograft tumors (C42 and C42BAD) were injected with either 10 μ l DMSO or 80 μ M LY294002, three times with 40 min intervals. Then, 40 min after last injection mice were anesthetized and tumors were excised. Akt phosphorylation was analysed by western blotting of whole cell lysates; HA-BAD was immunoprecipitated and probed with phosphospecific antibodies.
- B) Comparison of apoptosis induced by LY294002 and ZSTK474.

1. 5) Test whether stress or epinephrine injection will restore BAD phosphorylation in C42Luc xenografts treated with PI3K inhibitors.

Until we are successful in de-phosphorylating BAD in C42 xenografts we will not be able to reliably test whether stress/epinephrine signaling restores BAD phosphorylation in vivo.

2. Test if stress/epinephrine activates PKA and increases BAD phosphorylation in xenografts implanted in bones.

C42Luc tumors that grow in bones are mixed with mouse tissues (Fig. 4) Therefore the best way of selectively following BAD and CREB phosphorylation in tumors as oppose to stroma is by generating C42Luc cells that express BAD or CREB. We already generated C42LucBAD cells that express HA-BAD. HA-BAD could be selectively immunoprecipitated from tumors and phosphorylation at S112, S136 and S155 could be followed by Western blotting with phospho-specific antibodies (Fig. 3A). However unlike with subcutaneous xenografts it may not be feasible to directly inject PI3K inhibitors into xenografts implanted in bones. Therefore prior to experiments with xenografts implanted into bones we need to demonstrate that systemic injections of PI3K inhibitor de-phosphorylate Akt and BAD. For these experiments will use ZSTK474 that will be injected intraperitoneally into mice with subcutaneously implanted C42 xenografts. As positive control ZSTK474 will be injected directly into subcutaneous C42 xenografts. After effects of systemic injection of ZSTK474 on Akt and BAD de-phosphorylation will be demonstrated in mice with subcutaneous xenografts experiments will be continued with C42cells implanted in bones.

As alternative approach to pharmacological inhibition of PI3K we resort to inducible expression of PTEN lipid phosphatase in C42 cells. C42 cells do not express PTEN due to frame-shift mutation. Earlier publication has shown that re-expression of PTEN turns off

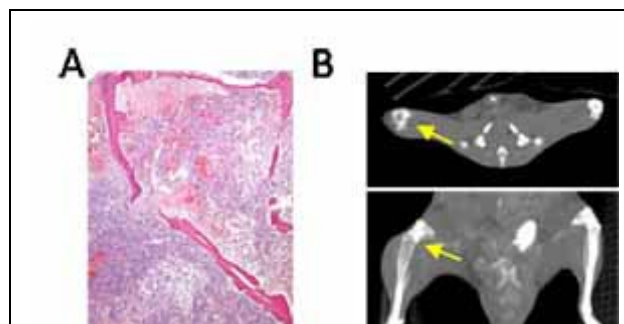


Fig. 4 C42 xenograft in fibia
A) Histochemical staining illustrates bone resorption and expansion of tumor outside the tibia.
B) Tibia resorption by C4-2Luc xenograft detected by CT scan

constitutive PI3K pathway in LNCaP cells (2). Since C42 cells were derived from LNCaP we anticipate that inducible expression of PTEN will lead to dephosphorylation of both Akt and BAD in C42 cells as well.

Key Research Accomplishments

1. We have shown that in tissue culture antagonists of β 2-AR inhibit epinephrine-induced activation of PKA and BAD phosphorylation as well as anti-apoptotic effect of epinephrine. This information opens doors to test the role of epinephrine/ β 2-AR in activation of PKA/BAD pathway in vivo.
2. We showed that intratumoral injections of LY294002 cause only transient (40 min) inhibition of PI3K/Akt pathway in C42 xenografts. Thus, repeated injections are required to achieve continuous inhibition of PI3K pathway.
3. We have shown that CREB phosphorylation correlates with increased blood epinephrine levels. Thus, it is necessary to measure blood epinephrine levels in order to confirm that mice either calm or stressed.

Reportable Outcomes

1. We generated C42Luc cells that inducibly express PKA inhibitor PKI-GFP.
2. We have submitted a manuscript that describes the role of BAD in prostate xenograft growth (see appendix).
3. We generated CRE-TSTA construct that could be used to monitor induction of CRE in tumors in vivo by non-invasive imaging.

Conclusion

We have reagents at hand to test the role of β 2-AR in stress-induced activation of PKA. PKA activity could be judged by phosphorylation of CREB and BAD. Although BAD phosphorylation is more relevant to anti-apoptotic effect of epinephrine, CREB could be used as biomarker of PKA activation. Still, additional experiments are needed to reliably monitor activation of PKA and BAD phosphorylation in vivo. Thus, in order to establish a connection between stress and PKA activation in tumors we need to condition mice so they will not be stressed by handling. We already know that repetitive handling is reducing stress response (based on measurements of blood epinephrine levels) and experiments are under way to establish the correlation between epinephrine levels and CREB phosphorylation.

As alternative approach we developing tools to monitor PKA activity by noninvasive optical imaging.

Since C42 cells are PTEN deficient, PI3K/Akt pathway is constitutively active and BAD is constitutively phosphorylated in these cells. Thus, to determine whether BAD could be phosphorylated by stress/epinephrine in vivo we need to inhibit PI3K in C42 xenografts. To accomplish this two approaches are under way: first, to use new PI3K inhibitor ZSTK474, second to generate cells that inducibly express PTEN phosphatase.

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Appendices

Manuscript "**Expression of the Bcl-2 protein BAD promotes prostate cancer growth**".

Title page

Expression of the Bcl-2 protein BAD promotes prostate cancer growth

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Running title:

BAD expression stimulates tumor growth

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This work was supported by NIH grant CA118329-01A1; DOD Grant PC073548, Grants from the
Comprehensive Cancer Center and WFUSM Interim Funding to GK; AS was supported by NIH
Award T32CA079448.

Abstract

BAD, a pro-apoptotic protein of the Bcl-2 family, has recently been identified as an integrator of several anti-apoptotic signaling pathways in prostate cancer cells. Thus, activation of EGFR, GPCRs or PI3K pathway leads to BAD phosphorylation and inhibition of apoptosis. Increased levels of BAD in prostate carcinomas have also been reported. It appears contradictory that instead of limiting expression of pro-apoptotic protein, prostate cancer cells choose to increase BAD levels while keeping it under tight phosphorylation control. Analysis of the effect of BAD on prostate cancer xenografts has shown that increased BAD expression enhances tumor growth, while knockdown of BAD expression by shRNA inhibits tumor growth. Tissue culture experiments demonstrated that increased BAD expression stimulates proliferation of prostate cancer cells. These results suggest that increased expression of BAD provides a proliferative advantage to prostate tumors, while BAD dephosphorylation increases sensitivity of prostate cancer cells to apoptosis. Combination of proliferative and apoptotic properties prompts prostate cancer cells to be “addicted” to increased levels of phosphorylated BAD. Thus, kinases that phosphorylate BAD are plausible therapeutic targets; while monitoring BAD phosphorylation could be used to predict tumor response to treatments.

KEYWORDS: prostate cancer; BAD; apoptosis; xenograft tumors; noninvasive optical imaging.

Introduction

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer-related deaths in men in the United States [1]. Currently there is no effective treatment for androgen-independent advanced prostate cancer [2]. Mechanisms that enable prostate cancer cells to evade apoptosis may contribute to therapeutic resistance. Thus, increased levels of several growth factors, including FGF, EGF, IL-6 and GPCR agonists that activate anti-apoptotic signaling pathways, have been reported in androgen-independent prostate cancer [3–7]. Anti-apoptotic signals could either post-translationally modify apoptosis regulatory proteins or change their expression levels. Indeed, increased expression of anti-apoptotic Bcl-2 proteins as well as inhibitors of apoptosis proteins (IAPs) in advanced prostate cancer has been reported [8,9]. Also, we have recently shown that in prostate cancer cells, the pro-apoptotic Bcl-2 protein BAD plays a unique role as a convergence point of several anti-apoptotic signaling pathways that include constitutively active PI3K, activated EGFR and GPCR [6].

BAD, bcl-xl/bcl-2- antagonist causing cell death, was initially identified in a yeast two hybrid screen interacting with Bcl-2 or Bcl-xl [10]. BAD is a unique BH3-only family member in that its regulation is primarily mediated through its conserved phosphorylation sites (serines 112, 136, and 155 based on the mouse sequence)[11,12]. Phosphorylated BAD fails to bind Bcl-XL or Bcl-2 proteins, and has been considered an apoptosis sentinel inactivated by anti-apoptotic signals. Upon withdrawal of survival factors BAD becomes dephosphorylated, shifts the balance of pro- and anti-apoptotic Bcl proteins that triggers release of cytochrome c, SMAC and AIF from mitochondria and subsequently leads to apoptosis [12]. Thereby, it would not be surprising if cancer cells attempted to decrease BAD expression.

1 A recent study has shown that BAD expression is elevated in prostatic carcinomas compared
2 to low expression in normal prostatic epithelium [13]. It seems counterintuitive that prostate cells
3 would dedicate extra resources to maintain BAD phosphorylation instead of eliminating its
4 expression. It is possible that in addition to regulating apoptosis, BAD might play a positive role in
5 prostatic tumor growth.

6 In this study, we found that increased BAD expression stimulates proliferation of prostate
7 cancer cells in tissue culture and prostate tumor growth *in vivo*. Thus, BAD expression may be
8 advantageous to prostate cancer cells. At the same time, BAD dephosphorylation increases
9 sensitivity of prostate cancer cells to apoptosis. This combination of proliferative and apoptotic
10 properties prompts prostate cancer cells “addiction” to increased levels of phosphorylated BAD
11 This scenario suggests that kinases that phosphorylate BAD are plausible therapeutic targets.

Materials and Methods

Cell lines – Prostate cancer cell lines, LNCaP and C4-2, were gifts from Dr. Leland Chung (Emory University, Atlanta GA). C4-2BADLuc cells were generated by transfecting C4-2 cells with wild-type BAD (HA-BAD-pTRE2hygro) and firefly luciferase (PGL3) whereas pTRE2hygro and firefly luciferase (PGL3) were transfected into C4-2 cells to generate C4-2Luc. LNCaP cells were maintained with T-medium supplemented with 5% fetal bovine serum, and C4-2 cells were maintained with RPMI 1640 with 10% fetal bovine serum. All cells were kept at 5% CO₂ at 37° C.

Antibodies and Other Reagents –Antibodies were obtained from the following sources: BAD, phospho-specific BAD (serines 112, 136, 155) from Cell Signaling Technology (Beverly, MA); ERK from Zymed Laboratories (South San Francisco, CA); secondary horseradish peroxidase-conjugated antibodies used for Western blots from Amersham Biosciences (Piscataway, NJ). All other chemicals (unless specified) were purchased from Sigma (St. Louis, MO). Tissue culture reagents were purchased from Invitrogen (Carlsbad, CA).

shRNA experiments - A lentiviral vector (pLL3.7) [14] was used with an shRNA insert of annealed oligonucleotides. The BAD DNA target sequences used were 5'-TGAAGGGACTTCCTCGCCCGT-3' and 5' GGCTTGGTCCCATCGGAAG-3'. HEK 293 cells were transfected with pLL3.7 vector containing either of these sequences or a scrambled sequence 5'-GGTACGGTCAGGCAGCTTCT-3' in combination with packaging vectors (VSVG, RSV-REV, and pMDL g/p RRE). After 48 h, supernatants were collected from these cells and used to infect LNCaP or C4-2 cells [6]. Forty-eight hours after infection, cells were plated for subsequent experiments.

1
2 *Proliferation Assays* – Cell counts were done by the following: 2×10^5 cells were plated in six cm
3 dishes for each experimental group. The initial cell count was 24 hours after cells had attached to
4 the dishes (Day 1). Two additional counts were made three days later (Day 4) and six days later
5 (Day 7) and then compared to the initial cell count. Counts were made by trypsinizing and
6 collecting cells in media, then manually counting on a hemacytometer. MTT assays were done by
7 plating cells in triplicate in 24 –well plates at varying densities.

8
9 *Immunohistochemistry* – Antibody staining was performed on histological sections of formalin-
10 fixed prostate tumor xenografts. Antigen retrieval was performed by heating slides at 95°C in
11 10mM sodium citrate buffer (pH 6.0) for 60 min. Then, sections were treated identically as follows:
12 1) incubated in 2% hydrogen peroxide to block endogenous peroxidase activity; 2) incubated with
13 blocking solution: 1% BSA, 0.1% tween20 in PBS (30 min, 25°C); 3) incubated with primary
14 antibodies, Ki-67 from Abcam Inc. (Cambridge, MA) diluted 1:25-1:200 in blocking solution
15 (overnight, 4°C); primary antibodies were followed by peroxidase conjugated anti-rabbit secondary
16 antibodies (10 µg/ml, in blocking solution, 30 min, 25°C) and revealed with 3-3'-diaminobenzidine
17 (DAB) as the developing chromogen. Between steps, specimens were washed in PBS 3 times.

18
19 *Subcutaneous Implantations* - Nude mice (BALB/cAnNCrj-nu from Charles River) received four
20 subcutaneous injections of 2×10^6 cells with Matrigel. Injections were made using an insulin syringe
21 and a 27 gauge needle. All manipulations with animals were conducted in humane manner, in strict
22 adherence with a protocol approved by institutional ACUC, which was designed to minimize
23 animal suffering.

1 *Luminescence Imaging* – Tumor growth was analyzed with a Xenogen IVIS[®] 100 optical imaging
2 system (Caliper Life Sciences, Hopkinton, MA). Animals were immobilized for substrate injection
3 and imaging through an attached gas anesthesia system consisting of 2% isoflurane/O₂. To account
4 for background and nonspecific luminescence, mice were imaged before injection of luciferase.
5 Animals were injected with 100µl of the firefly luciferase substrate luciferin (3.5 mg/ml in PBS)
6 and imaged 15 minutes later in prone and supine positions (5 minutes each). Whole-body images
7 were obtained using the Living Image[®] software provided with imaging system. A gray-scale
8 photographic image and the bioluminescent color image are superimposed to provide anatomic
9 registration of the light signal. A region of interest (ROI) was manually selected over the
10 luminescent signal, and the intensity was recorded as photons/second within an ROI.

11
12 *Statistical analysis* - To determine whether differences between data sets were statistically
13 significant, Student's t-test analysis (two-tailed distribution; two-sample unequal variance) was
14 performed using Excel software.

1 Results

3 BAD expression stimulates proliferation of prostate cancer cells

4 Reports on increased expression of BAD in prostate cancer led us to suggest that prostate
5 cancer cells may benefit from maintaining BAD expression. To address the possible role of
6 increased BAD expression, we examined proliferation of prostate cancer cells that overexpress
7 BAD. For this purpose, we compared proliferation of cell lines that ectopically express BAD and
8 cell lines transfected with empty vector. Cells with elevated levels of BAD were characterized by
9 increased proliferation in tissue culture (Fig. 1A, B). To exclude the possibility that increased
10 proliferation of cells that stably express BAD was due to clonal variations, we compared
11 proliferation in cells transiently transfected with either BAD or empty vector. These experiments
12 showed increased proliferation in several cell lines that transiently express BAD (Fig 1C).

13 Since C4-2 cells are derived from metastatic prostate cancer [15], it is possible that they may
14 have already established optimal levels of BAD. Therefore, we examined the effect of knocking
15 down BAD expression on proliferation of these cells. C4-2Luc cells were infected with lentiviral
16 vectors that encode scrambled shRNA or BAD shRNA. Reduced expression of BAD led to
17 decreased proliferation of C4-2 cells (Fig. 1 D, E).

19 BAD expression stimulates prostate tumor growth

20 Experiments in tissue culture have shown that expression of BAD stimulates division of prostate
21 cancer cells as well as other cancer cells. To determine whether increased expression levels of
22 BAD stimulate prostate tumor growth *in vivo*, we compared growth of C4-2Luc and C4-2LucBAD
23 cells implanted in immunocompromised mice. C4-2Luc and C4-2LucBAD cells express firefly
24 luciferase that allows the monitoring of xenograft growth noninvasively by optical imaging.

Measuring luminescence instead of physical tumor size permits detection of xenograft growth prior to the appearance of palpable subcutaneous tumors. This approach reduces the time required to measure kinetics of tumor growth. Analysis of luminescence of C4-2Luc and C4-2LucBAD xenografts showed increased tumor take and faster tumor growth of C4-2LucBAD xenografts (Fig. 2AB). Consistent with results of luminescence analysis, C4-2LucBAD cells produced palpable tumors at higher frequency comparing to C4-2Luc cells (Fig. 2C). In accordance with the faster growth of C4-2LucBAD xenografts, immunohistochemical analysis showed an increased number of cells that stained positive for the proliferative marker Ki-67 compared to C4-2Luc xenografts (Fig. 2D).

Knockdown of BAD expression by shRNA inhibits tumor growth.

Parallel to experiments with C4-2LucBAD cells, experiments were conducted with C4-2Luc cells in which endogenous BAD expression was inhibited by the shRNA approach. C4-2Luc cells were infected with the lentiviral vector pLL3.7 that expressed BAD shRNA or scrambled shRNA, and the cells were then implanted subcutaneously into nude mice. Luminescence of C4-2Luc xenografts was followed for one week as shown in Fig. 3. C4-2Luc xenografts with a reduced expression of BAD showed reduced tumor take and grew at a slower rate than cells with intact BAD expression.

1 Discussion

2 *The novel role of BAD in promoting tumor growth.*

3 The results presented in this paper show that BAD, the BH-3 only Bcl-2 protein, might
4 function in a dual capacity in prostate cancer. When dephosphorylated, BAD promotes apoptosis
5 [11], while in a phosphorylated form it stimulates proliferation and tumor growth *in vivo*. This
6 connection between BAD expression and proliferation provides a possible explanation for an
7 increase in expression of phosphorylated BAD protein in prostate tumors.

8 Recently, several studies have shown that functions of BAD may extend beyond sensitizing
9 cells to apoptosis. For instance, publications from the Peter Vogt and Elizabeth Yang laboratories
10 have suggested that BAD protein can be involved in promoting cell cycle progression [16,17].
11 Thus, fibroblasts with increased expression of Bcl-2/BclXL are characterized by reduced apoptosis
12 and also by decreased proliferation. However, when Bcl-2 or BclXL forms a heterodimeric
13 complex with BAD, cells can overcome the G0/G1 growth arrest and enter into S phase [17,18].
14 These findings were extended to T cells by showing that T-cells over-expressing BAD were more
15 likely to remain in S-phase [19].

16 In other recent reports, BAD in the phosphorylated form was found to promote assembly of
17 active glucokinase complexes, an initial step of the glycolytic pathway [20,21]. Although both
18 increased proliferation and glycolysis are hallmarks of tumor growth, the experimental evidence
19 that connects BAD expression with tumor growth has been lacking.

20 *Could BAD play a dual role in prostate cancer cells?* Several reports have shown that cells that
21 express BAD proliferate faster; however, mechanistic details on how BAD promotes proliferation
22 diverge. One possibility is that BAD provides a counterbalance for increased levels of BclXL and
23 Bcl-2 that are known to slow proliferation [22]. If this scenario is correct, any pro-apoptotic
24 Bcl2/BclXL antagonist would be expected to have a BAD-like effect. However, if expression of

1 such an antagonist is constitutive, it would defeat the purpose of increased Bcl-2/BclXL expression
2 by increasing apoptosis sensitivity. Since the proportion of BAD that could form heterodimers with
3 anti-apoptotic counterparts depend on phosphorylation status, BAD may be uniquely suited for the
4 role of modulator of Bcl2/BclXL, availability of which is fine tuned by protein kinases. It is also
5 possible that by increasing rate of glucose utilization, BAD expression provides competitive
6 advantage to the cells in hypoxic tumors that switch from oxidative phosphorylation to glycolysis
7 [23].

8 The precise mechanism of how Bcl proteins regulate proliferation is obscure. It remains to
9 be determined whether a single mechanism plays a dominant role or BAD-dependent stimulation of
10 proliferation is mediated via several mechanisms simultaneously, and whether BAD localization to
11 a specific organelle (e.g. mitochondria, ER, nuclear envelope) is important. Also, this positive
12 effect on cell division may not be uniformly manifested in all cancer cells. Thus, BAD reportedly
13 inhibits G1 to S transition in MCF7 breast cancer cells [24]. Until the effects of BAD on
14 proliferation are dissected on a molecular level, we remain with the notion that effects of BAD
15 expression on proliferation are cell type-dependent.

16 Conclusions

17
18
19 Regardless of the exact mechanism that permits BAD to stimulate tumor growth, this
20 capacity may provide selective pressure to increase BAD expression in tumors. Activation of
21 protein kinases that phosphorylate BAD creates a permissive condition to increased expression of
22 BAD. It is tempting to speculate that that high BAD expression should make these tumors
23 increasingly sensitive to inhibitors of signaling pathways that control BAD. If so, high levels of
24 phosphorylated BAD could be used to identify patients who will benefit from therapy with such
25 inhibitors. Future studies in animal models and analysis of clinical trials data with regard to BAD

1 expression/phosphorylation are needed to determine the translational value of extensive efforts
2 spent studying protein kinases that phosphorylate BAD.

3 Acknowledgements

4 We are grateful to James Wood for FACS analysis, and to Karen Klein for editing.

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Figure legends

Figure 1. BAD expression increases proliferation of prostate cancer cells.

(A) HA-BAD expression in C4-2LucBAD clone.

(B) C4-2LucBAD cells proliferate at a faster rate than C42 cells. C4-2LucBAD cells or C4-2Luc cells were plated in triplicate 6 cm dishes. At days 1 and 4 after plating, cells were trypsinized and counted. Results at 4 days were significantly different with a p value of <0.001. Comparable results were obtained in experiments in which proliferation was measured with the MTT assay.

(C) Transient expression of HA-BAD stimulates proliferation. LNCaP cells were transfected with 9:1 mixture of GFP and either of HA-BAD or empty expression vector. Seven days after transfection, the number of GFP positive cells was counted. Graph shows the HA-BAD/empty vector ratio of GFP positive cells. Proliferation of GFP-positive cells was confirmed by time lapse video recording.

(D) Knocking down BAD expression with shRNA decreases proliferation. A lentiviral vector (pLentiLox 3.7) with a BAD shRNA insert was used to infect C42 cells. C4-2Luc cells were plated in triplicate 6 cm dishes. At day 1 and 4 after plating, cells were trypsinized and counted. Experiments were repeated at least 3 times.

(E) Western blot analysis of BAD expression in cells infected with empty lentiviral vector, scrambled shRNA or BAD-specific shRNA. Expression of total ERK was used as loading control.

Figure 2. Over-expression of BAD increases tumor growth rate and tumor take.

Nude mice received four subcutaneous injections of 2×10^6 C4-2Luc or C4-2LucBAD cells.

(A) Representative whole body images of the animals obtained at 1 day and 2 weeks after implantations using the IVIS100 and Living Image® software (Xenogen).

(B) Dot plot showing fold increase and median luminescence in mice injected with C4-2Luc and C4-2LucBAD cells.

1 **C)** Percent of palpable tumors (over 5 mm) developed at injection sites.

2 **D)** Representative tissue sections of formalin-fixed tumors stained for proliferation marker Ki67.

3

4 **Figure 3. Knocking down of BAD expression with shRNA inhibits growth of prostate cancer**
5 **xenografts.** Nude mice received two subcutaneous injections of 2×10^6 C4-2Luc cells infected with
6 lentiviral vector with BAD shRNA (right side) or an empty vector (left side). Images and
7 quantification are as in Figure 2.

8 **A)** Representative images of C4-2Luc and C4-2LucBAD tumors.

9 **B)** Dot plot shows the ratio between luminescence at one week/ day 1. After eight weeks, palpable
10 tumors were detected only at sites injected with C4-2Luc cells infected with empty vector.

Figure 1

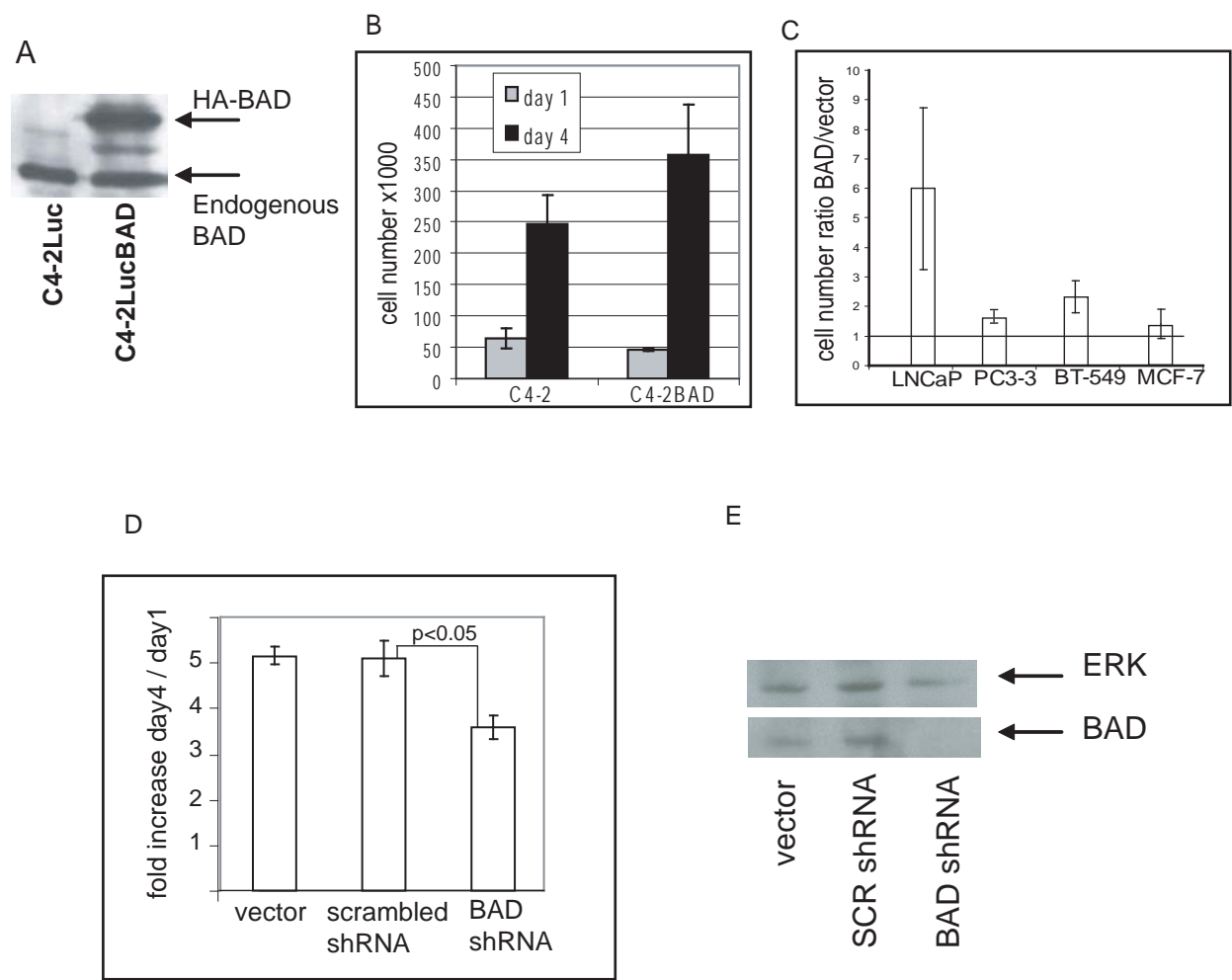


Figure 2

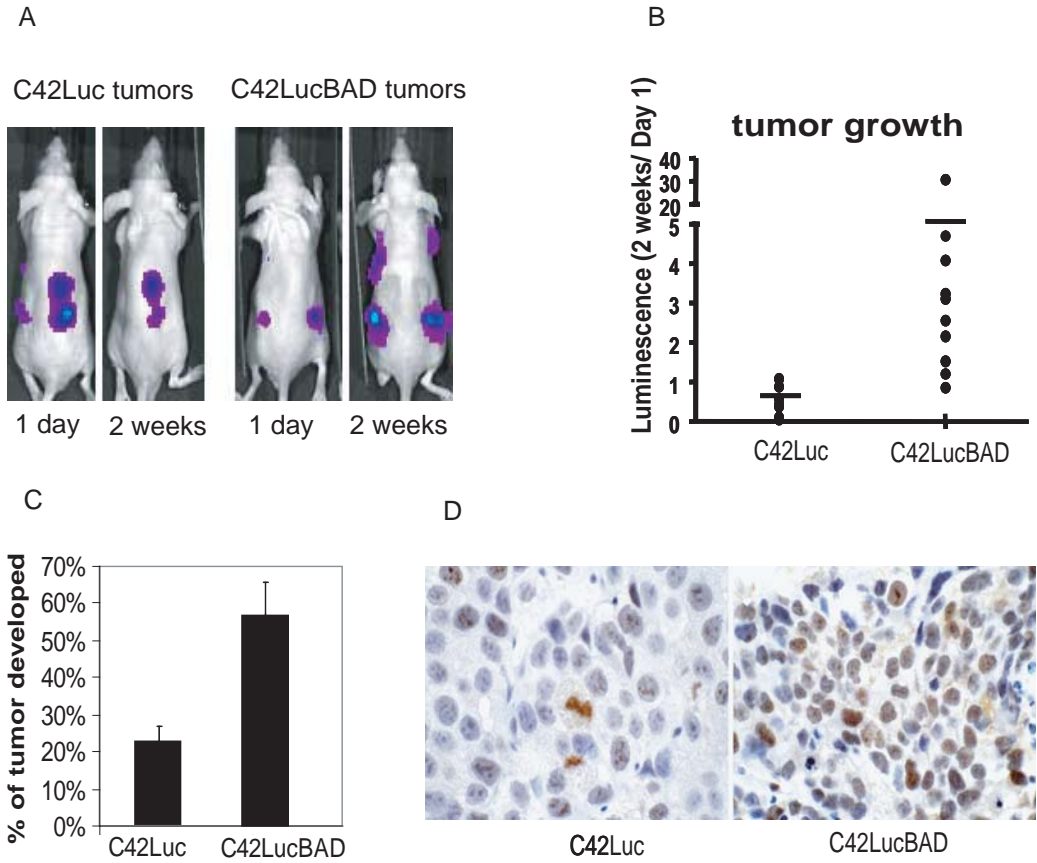


Figure 3

